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New insights into the melanophilin (*MLPH*) gene controlling coat color phenotypes in American mink

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ABSTRACT

The mutation causing the *Silverblue* color type (*pp*) is one of the most used recessive mutations within American mink (*Neovision vision*) fur farming, since it is involved in some of the popular color types such as *Violet* and *Sapphire* which originate from a combination of recessive mutations. In the present study, the genomic and mRNA sequences of the melanophilin (*MLPH*) gene were studied in *Violet*, *Silverblue* and wild-type (*wt*) mink animals. Although breeding schemes and previous literature indicates that the *Violet* (*aammpp*) phenotype is a triple recessive color type involving the same locus as the *Silverblue* (*pp*) color type, our findings indicate different genotypes at the *MLPH* locus. Upon comparison at genomic level, we identified two deletions of the entire intron 7 and of the 5' end of intron 8 in the sequence of the *Silverblue MLPH* gene. When investigating the mRNA, the *Silverblue* animals completely lack exon 8, which encodes 65 residues, of which 47 define the Myosin Va (*MYO5A*) binding domain. This may cause the incorrect anchoring of the *MLPH* protein to *MYO5A* in *Silverblue* animals, resulting in an improper pigmentation as seen in diluted phenotypes. Additionally, in the *MLPH* mRNA of *wt*, *Violet* and *Silverblue* phenotypes, part of intron 8 is retained resulting in a truncated *MLPH* protein, which is 359 residues long in *wt* and *Violet* and 284 residues long in *Silverblue*. Subsequently, our findings point out that the missing actin-binding domain, in neither of the 3 analyzed phenotypes affects the transport of melanosomes or the consequent final pigmentation. Moreover, the loss of the major part of the *MYO5A* domain in the *Silverblue MLPH* protein seems to be the responsible for the dilute phenotype. Based on our genomic DNA data, genetic tests for selecting *Silverblue* and *Violet* carrier animals can be performed in American mink.

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1. Introduction

The mutation causing the *Silverblue* color type is one of the most used recessive mutations within mink fur farming. It is used in homozygous form, such as in pure *Silverblue* (*pp*) or is part of some of the popular color determining genotypes in which several recessive mutations are combined, such as for instance in the light sand color type *Pearl* (*ppmm*), *Violet* (*aammpp*) and the *Bluish Sapphire* (*aapp*). In the latter two phenotypes, also the *LYST* gene is involved

(Anistoroaei et al., 2013). The *Silverblue* phenotype on a wild type mink background is characterized by a bluish gray color, with slightly darker tail and feet in comparison to the rest of the body (Fig. 1A). The nose and eyes are lighter in color than the wild-type (*wt*) (Fig. 1C). *Violet* (also known as *Moyle Sapphire*) is a pale blue type with red eyes (Fig. 1B) and is less brownish in comparison to the *Silverblue* phenotype.

As previously reported, the *Silverblue* mink coat color is inherited as a Mendelian autosomal recessive trait and has been determined to involve the *MLPH* gene (Anistoroaei and Christensen, 2007). In the mink genetics literature (Clausen et al., 2012) two alleles are described at the *Silver* locus; *Silverblue* (*pp*) and *Steelblue* (*p^sp^s*). Important to mention is the fact that until now, none of the classical *a* to *e* color genes have been identified in the American mink; therefore the mink genetics have developed an independent nomenclature (Clausen et al., 2012).

The dilution phenotype for coat color occurs in many mammalian species and is characterized by a defective transport of melanosomes to the melanocytes, leading to large clumps of pigments around the perinuclear region and resulting in a lighter coat color of the animal

Abbreviations: *MLPH*, melanophilin; *pp*, *Silverblue* genotype; *wt*, wild-type; *aammpp*, *Violet* genotype; *MYO5A*, Myosin Va; mRNA, messenger RNA; *ppmm*, *Pearl* genotype; *aapp*, *Bluish Sapphire* genotype; *LYST*, lysosomal trafficking regulator gene; *p^sp^s*, *Steelblue* genotype; *RAB27A*, member RAS oncogene family; SNP, single nucleotide polymorphism; PCR, Polymerase Chain Reaction; ng, nanograms; BLAST, Basic Local Alignment Search Tool; cDNA, Complementary DNA; mg, milligrams; Sdw, sterile distilled water; BAC, Bacterial artificial chromosome; UTR, untranslated region; nt, nucleotides; bp, base pair; LOD, logarithm (base 10) of odds; G, Glutamine; H, Histidine; S, Serine; N, Asparagine; ORF, open reading frame; NCBI, National Center for Biotechnology Information; DeltaAB, *Slac2-a* deletion mutant; KA, *Slac2-a* point mutant.

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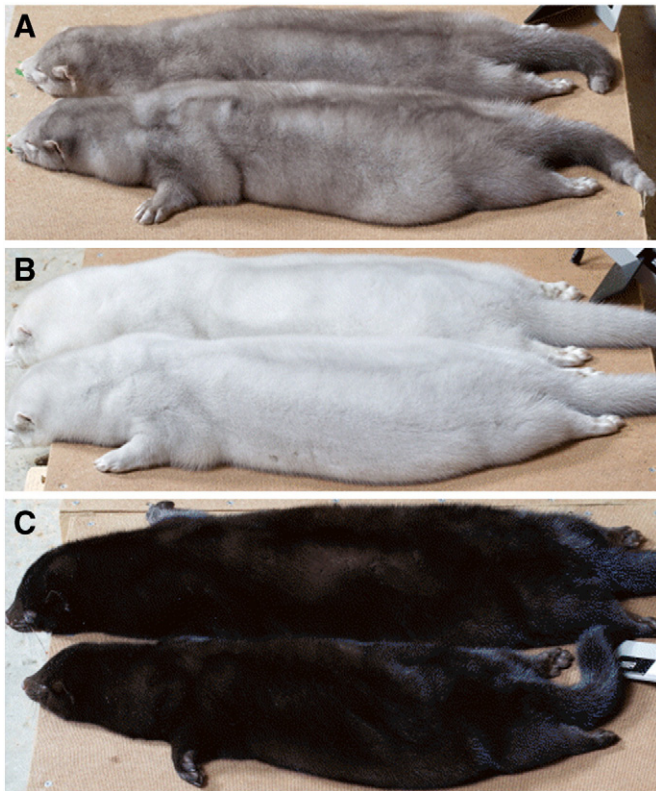


Fig. 1. A. Silverblue mink; B Violet mink; C wt mink.

(Barral and Seabra, 2004). The molecular biology behind pigmentation is rather complex, involving many genes (Passeron et al., 2005). Mutations in three different genes i.e. *Myosin Va* (*MYO5A*), *RAB27A* and *MLPH*, have been described to be responsible for the diluted phenotype (Hume et al., 2007). The products of these three genes collaborate to anchor the melanosomes to the actin cytoskeleton (Nascimento et al., 2003), forming a protein–actin complex which directs the transport of the melanosomes within the cell. In mouse, three coat color mutants: *dilute*, *ashen* and *lead* have been characterized, in which the abovementioned three genes are involved (Matesic et al., 2001; Mercer et al., 1991; Wilson et al., 2000). In humans, mutations in the *MLPH* gene have been shown to cause the Griscelli syndrome type III, an autosomal recessive disorder characterized by pigment dilution of the skin and hair (OMIM 609227) (Menasche et al., 2003). In cats, a single base pair deletion in exon 2 of the *MLPH* gene causes the feline dilute coat color phenotype (Ishida et al., 2006), while in dogs, a SNP at the splice donor site of exon 1 of the *MLPH* gene has been described as a candidate causal mutation for coat color dilution (Drögemüller et al., 2007). Similarly, in poultry, Bed'hom et al. (2008) found a large deletion in the *MLPH* gene region in lavender quails. More recently, Vaez et al. (2008) have reported a causative non-synonymous single nucleotide polymorphism (SNP) (R35W) in the *MLPH* gene, on the basis of the lavender phenotype in chickens.

The *MLPH* gene is relatively well conserved between species. A *MLPH* protein consists of three domains of which two are very important: the *RAB27* similar domain, which attaches the *MLPH* to the *RAB27* protein at the surface of the melanosome, and the *Myosin Va* (*MYO5A*) domain, which binds specifically the *MLPH* to the *Myosin*. In human and mice, it is postulated that the dilution phenotypes are caused by the inability of *MLPH* to bind *RAB27* and link it to *MYO5A*. As the *MLPH* sequence in wt mink has been generated (Anistoroaei et al., 2011; Acc: JF288169) we were able to sequence and compare the region between the *Silverblue*, the *Violet* and the *wt* phenotypes in

mink at genomic- and mRNA levels. We discuss the functional implications of the different mutations found in the light of coat color phenotypes in American mink. Furthermore, genetic tests are proposed to select for carriers of *Silverblue* and *Violet* phenotypes in mink.

2. Material and methods

2.1. Pedigree and genotyping

A half-sib family originating from a Danish farm in Sealand, Denmark, with 22 offspring that segregated for the *Silverblue* color described in Anistoroaei and Christensen (2007; Supplementary Fig. 1A) was genotyped with three new microsatellite markers: *RAN79*, *RAN80* and *RAN81* (Table 1). These markers were developed from the assembled contig (#JF288169) containing the *MLPH* gene (Anistoroaei et al., 2011). Additionally, a half-sib family originating from the Taastrup Experimental farm of the Faculty of Life Sciences, University of Copenhagen, Denmark, with 22 offspring that segregated for the *Violet* color was genotyped with the same markers. Briefly, genomic DNA was extracted from tongues of the euthanized mink by using a Promega DNA extraction kit (#A1125). DNA amplification was performed with fluorescently labeled forward primers (HEX and NED) on an ABI Prism 3130 sequencer, and genotypes were analyzed using Genscan analysis (V.3.1.2) software (Applied Biosystems). The dilute animals were classified as homozygous for the recessive mutated dilute allele (*pp*) and the other types of colored mink were classified as carrying at least a single copy of the dominant wild-type allele (*P*).

2.2. Sequence analysis of the *MLPH* gene

The genomic contig containing the *MLPH* gene (#JF288169) was analyzed in silico in order to predict the exonic sequences of the *MLPH* gene with human, mouse, dog and cat sequences as references. Furthermore, transcriptome data from a *wt* mink (Anistoroaei et al., 2012a) was used to identify the possible transcripts of the *MLPH* gene. The analyses were performed by using Spidey software (<http://www.ncbi.nlm.nih.gov/spidey/>).

Primers were designed from the flanking intronic regions of all predicted exons of the *MLPH* gene of mink (#JF288169; Anistoroaei et al., 2011) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). As the genetic material from the *Violet* family was freshly prepared, we started our sequencing analysis based on *Violet* instead of pure *Silverblue*. A total of 15 primer pairs were designed and used to amplify each of the 16 exons and an upstream region of the *MLPH* gene in *wt* and *Violet* (see Table 2 for primer sequences). PCRs were carried out at standard conditions at 60 °C annealing and 2 mM magnesium. In order to verify the SNPs found in exon 8 and intron 8, also 5 *Violet* and 2 *Silverblue* animals from the original study (Anistoroaei and Christensen, 2007) as well as 2 *wt* animals were sequenced for this region. PCR products were cleaned using a PCR purification kit (Qiagen, Germany) and quantified by NanoDrop ND-1000 spectrophotometer (Thermoscientific, USA). Subsequently, 40–80 ng of DNA was used for each sequencing reaction using BigDye terminators (Applied Biosystems) following the

Table 1
Microsatellite markers used for genotyping the *Silverblue* and *Violet* mink families.

Primer pair name	Forward and reverse primers (5'3')	Repeat motif	Alleles size
<i>RAN79</i>	F: CCTGCAGACAGTGAGAGCTG R: CAACCCCTTGGGAAAATAC	(GT) ₂₂	200–210
<i>RAN80</i>	F: CCAGCCAAGGATCTAAGCAC R: GTCGGGCTCCCTTTATTCTC	(CT) ₁₂	235–241
<i>RAN81</i>	F: GGGATTCTCTCTCCCTCACC R: GGCTACATTCAAGGACACA	(TC) ₁₃	199–205

manufacturer's instructions. The sequencing reactions were cleaned by a Millipore system and sequenced using a 3130XL sequencing machine (Applied Biosystems). DNA sequences were processed by means of Lasergene (DNA Star Inc.), and they were compared using BLAST *bl2seq*.

2.3. RNA isolation, cDNA synthesis and cDNA sequencing

Four *Silverblue* and four *wt* mink puppies were euthanized and several tissues were collected including skin, lung and kidney and placed at -80°C until use. For cDNA preparation, around 100 mg of tissue was used for total RNA isolation using the TRI Reagent® protocol according to the manufacturer's recommendations. Homogenization was performed using a gentleMACS™ Octo Dissociator machine (Milteny Biotec, GmbH, Germany). Remaining genomic DNA was degraded using the RNase-free DNase step from the RNeasy micro kit (Qiagen, Germany). Quantity and quality of the isolated RNA was measured in a NanoDrop ND-1000 (Thermoscientific, USA) and visually inspected after running it in a 1.4% agarose gel. All RNA samples were kept at -80°C until cDNA synthesis. Briefly, one μg of total RNA from each tissue and animal was reverse transcribed at 42°C using Improm-II™ reverse transcriptase (Promega) and a mixture 3:1 of random hexamers: oligodT, according to the manufacturer's recommendations. The four cDNA samples for each phenotype and tissue were pooled and diluted 1:8 in RNase-free Sdw prior to performing

the PCR. Additionally, available cDNA from lung of a *Violet* animal was included in the study.

Primer sequences for the region encompassed by exon 7 to exon 9 were designed using Primer3 software (<http://frodo.wi.mit.edu>; Rozen and Skaletsky, 2000) (Table 2) and used in different combinations. The PCR products resulting from amplification of kidney and lung cDNAs (from both *wt* and *Silverblue*) contained more DNA and were selected for sequencing. PCR products were cleaned and sequenced as described above.

3. Results

3.1. The mink MLPH gene as derived from the BAC contig

Based on the analysis of the assembled genomic contig containing the *MLPH* gene (#JF288169), derived from the CHORI-231 *wt* color American mink BAC library (Anistoroaei et al., 2011), the mink *MLPH* gene (UTRs and coding regions) is predicted to be represented by 16 exons and 2450 nucleotides (nt). Based on in silico comparisons within mink, human, mouse, cat and dog sequences, the coding region of the *MLPH* gene virtually consists of 1719 bp, which theoretically would translate into 573 amino acids. However, the results of the present study (see description below) indicate that the *MLPH* transcript in *wt* mink is only 1077 bp long, yielding a protein of 359 amino acid residues (Fig. 2).

3.2. Genotyping of the mink families

The genomic contig of the *MLPH* gene (#JF288169) provided 21 di-, tetra-, penta- and hexa-nucleotide repeated motifs, which could subsequently be developed into microsatellite markers. Microsatellite markers *RAN79*, *RAN80* and *RAN81* were further developed and characterized to be used in this study.

The new markers fully confirmed the association to the *Silverblue* phenotype. In addition, in another study, *RAN79* was linked to chromosome 3 and in situ hybridized to American mink 3q1.3-2.2 (Anistoroaei et al., 2012b). The genotyping of the 3 above mentioned markers for animals within the family segregating for the *Silverblue* phenotype (Supplementary Fig. 1) revealed informative polymorphisms for all of them, each of them yielding a LOD score value of 4.52 and perfect co-segregation with the *Silverblue* phenotype. The markers were not informative for the *Violet* family.

3.3. Analysis of the genomic sequence of the *MLPH* gene in *Silverblue*, *Violet* and *wt*

Sequencing the region encompassed from exon 7 to exon 9 in the gDNA of 2 *Silverblue* animals revealed the complete loss of intron 7 and the first part of intron 8 (from position 1 to position 2224). The same genomic regions in *wt* and *Violet* revealed no insertion/deletion and only three SNPs: 1. a non-synonymous SNP segregating in both the *Violet* and *wt* phenotypes is located in exon 8 (c-785G>C), which results in an amino-acid variation in the *MLPH* protein (from Glutamine (G), an uncharged polar residue, to Histidine (H), a basic residue); 2. a synonymous SNP in exon 8 (c-885G>A) where both *Violet* and *wt* are heterozygous and 3. one SNP at the acceptor site of exon8/intron8, where the G nucleotide at the splice site of the *wt* is mutated to an A in the *Violet* (c.901+1G>A) (Fig. 4).

The rest of the exons and 5' UTR region of the *MLPH* gene were sequenced in *Violet* and *wt*. We identified three SNPs and one insertion: two of the SNPs occur in a region located approximately 5500 nucleotides upstream of the *MLPH* gene (c-5706T>C and c-5703A>G) where we also found a 5 nucleotides insertion in *Violet* (a 5847CATTT sequence in *wt* compared to 5847GGGGTAGGAA in *Violet*); the third SNP segregates in both the *Violet* and *wt* phenotypes at position 10 of exon 2 (c-11T>G) (see Fig. 4).

Table 2
Primer sequences and amplicon size for all *MLPH* exons.

Gene name	Primer sequences	Amplicon size
<i>UpMLPH</i>	fw: 5' TCACTCTCTTCTGGCTTGACTT 3' rev: 5' CATTTTCAAACAGAGCTCT 3'	563 bp
<i>MLPH.Exon1</i>	fw: 5' TGTTCAAAAAGCTCTAAAAGCCTCC 3' rev: 5' GGTATTATAGAGGGCAGAGCTTGCA 3'	492 pb
<i>MLPH.Exon2</i>	fw: 5' TTAGAAAAGCATTACACCCAGGTCC 3' rev: 5' GCATTGATGCTTCTGCTGCTCTG 3'	368 bp
<i>MLPH.Exon3–4</i>	fw: 5' TGCCCTGGGGTGACTTACCTGT 3' rev: 5' TGGGAACAAGAGCCAGGCC 3'	694 bp
<i>MLPH.Exon5</i>	fw: 5' CTCGCCGTGACATCCACAGTA 3' rev: 5' TCGCCGACTCTTACATCTC 3'	356 bp
<i>MLPH.Exon6</i>	fw: 5' CAATCTCTCTCCCTGGGTTG 3' rev: 5' GACCGGGGAAGACTTACTTTG 5'	164 bp
<i>MLPH.Exon7</i>	fw: 5' GCCCTTAGTGCTGCCITGA 3' rev: 5' TGGCCGTCAAACCTCTTCCC 3'	380 bp
<i>MLPH.Exon8</i>	fw: 5' TCACGGGACACATGATACAGAGTG 3' rev: 5' ACCCTTCCCAATGCAGAG 3'	433 bp
<i>MLPH.Exon9</i>	fw: 5' GTGCACTGGCTTCCAACTTT 3' rev: 5' AAAGCATCAGCAGGAAGGAA 3'	242 bp
<i>MLPH.Exon10</i>	fw: 5' CACACAGAGGACCAGCTGAA 3' rev: 5' AGGCAGCCTCACCTCCAG 3'	242 bp
<i>MLPH.Exon11</i>	fw: 5' GGCCCAACAAGAGCTGCAT 3' rev: 5' AGCTTTGGTGCACTGTCCACAG 3'	396 bp
<i>MLPH.Exon12</i>	fw: 5' CTGCATGGGGGTGATCCTTGA 3' rev: 5' TGTTCCAGTCACACCCAGCCAG 3'	432 bp
<i>MLPH.Exon13</i>	fw: 5' AAACAAGCCACCGTCAGTA 3' rev: 5' GCTGAGCTGCAAGGTGTGT 3'	373 bp
<i>MLPH.Exon14</i>	fw: 5' TGGTGTTCATTATCGATACCGTG 3' rev: 5' CAGTGCAGAGAATCCAGAACACC 3'	444 bp
<i>MLPH.Exon15</i>	fw: 5' AGACGGCTGCTCTGCGGACT 3' rev: 5' TCCATTTCCTCTGCTCTGG 3'	196 bp
<i>MLPH.Exon16</i>	fw: 5' TGGTGTGTGTGTTCAATGAACCG 3' rev: 5' GCCAAAAAGAACTGATCCCTTT 3'	390 bp
<i>Exon7. MLPH^a</i>	Fw: 5' TTTGAGGCTGACTCTGACGA 3'	
<i>Intron7. MLPH^a</i>	fw: 5' TCACGGGACACATGATACAGAGTG 3'	
<i>Exon8. MLPH^a</i>	fw: 5' GTGTGCTCAAACCTTCATGG 3'	
<i>Exon8. MLPH^a</i>	rev: 5' CAGGGCTGTCTGCGGACT 3'	
<i>Intron8. 1. MLPH^a</i>	rev: 5' ACCCTTCCCAATGCAGAG 3'	
<i>Intron8. 2. MLPH^a</i>	rev: 5' CTGAACCTCTGAACCCACAC 3'	
<i>Exon9.1. MLPH^a</i>	rev: 5' GCACTGAACAGCCACAGTA 3'	
<i>Exon9.2. MLPH^a</i>	rev: 5' TGGTGGAGTATTGCTCTCTT 3'	

^a Extra primers sequences designed for the region from exon 7 to exon 9.

	1
human	MGKKLDLSKLTDEEAQHVLVQVQDFDLRRKEERLEALKGKIKKESKRELLSDTAHLN
mouse	MGRKLDLSTLTDEEAHVWVQVQDFDLRRKEERLQGLKGKIQKESKRELLSDTAHLN
dog	MGKKLDLSKLTDEEAHVWVQVQDFDLRRKEERLEGLKGKIKKESQRELLADSAHLN
mink (wt)	MGRKLDLSKLTDEEAHVWVQVQDFDLRRKEERLEGLKGRIKKESSQRELLADTAHLN
	:.*****:* *****:*****:***:***:***:***:***:***
human	ETHCARCLQPYQLLVNSKRQCLECGFTCKSCGRVHPPEEQGWICDPCHLARVVKIGSLEW
mouse	ETHCARCLQPYRLLLNSRRQCLESFLVCKSCSHAHPEEQGWLCDPCHLARVVKIGSLEW
dog	ETHCARCLQPYRLLVTPKRQCLDCHLFTCQDCSHAHPEEQGWLCDPCHLARVVKIGSVEW
mink (wt)	ETHCARCLQPYRLLATPRRQCCLDCLFTCQNCNGHAHPEEQGWLCDPCHLARCVKIGSLEW
	*****:***.***:***:* ***:***:***:***:***:***:***:***:***:***
	146
human	YYEHVKARFKRFGSAKVIIRSLHGRLLQGGG-----GPELISEERSGDSQDTE
mouse	YYQHVRAFRFKRFGSAKVIIRSLCGRLLQGGG-----GSEPSLEENGNDSEQDTE
dog	YHKHLRARFKRFGSAKVIIRSLCGRLLQAGSPDSMRSSPDNHSPEPSPGSGSDSEQDTE
mink (wt)	YHQHLRARFKRFGSAKVIIRSLCGRLLQAGGEPDRARGSPDIHSGPEASTGEGSGDSEQDTE
	*:***:***** ***** ***.*** **
human	DGEPGSEAQAQAPFGS-KKKRLLSVHDFDFEGSDSDSTQPGHSLHLSSVPEARDSFQS
mouse	DGDLDTA--RDQPLNSKKKRLLSFRDVFEESSDHLVQPCSQTLGLSSVPESAHLQS
dog	DGELGTVA--QAQPLGS-TKKR-LSFHDLDFAEDSDSTWSGSHPPHSSPVSVATDSLQV
mink (wt)	DGELDTAA--QAQPIGS-KKKR-LSIHDLDFEADSDSTWSGCHPDSSPVAATDSLQV
	:*:*:*:* *:***:***:***:***:***:***:***:***:***:***
	241
human	LTDESC---SEKAAPHKAEGLEEADTGASGCHSHPEEQPTSPSRHGALAEELCPGGGS
mouse	LSGEPY---SEDTTSLPEEGLEETGARALGCHPSPEVQPCSPPLSGEDAHAELDSPAAS
dog	PCAQALTHGPRADASQEAALVEADVAAGCRAHPPEEQMDSLSAPGRDALAEPCLPAGS
mink (wt)	PCAQTFMDEPRAKDASPEAM-----VAPGCQPPPEEQMDSLSVPGQDTLTELCPGGS
	: . : * ***:***:***:***:***:***:***:***:***:***
human	HRMALGTAAALGSNVIRNEQLPLQYLADVDTSDE--ESIRAHVMASHHKKRRGRASSESQ
mouse	CKSAFGTTAMPGTDDVRGKHLPSQYLADVDTSDE--DSIQGPRAASQHSKKRRARTVETQ
dog	CSTA----AAGTHAIGREPLRSQHLADADISVSEDEGTGAPGTTSQHPRRGHTPAESQ
mink (wt)	RRTALGTAAAGRSITGSEQLPSQYLADMDTSEDDGESPRAPHAASHPCRGGGHTPQSQS
	* * : * * :*** * * . . . : * : : . : : *
human	GLGA-----GVRTEADVEEALRRKLEELTSNVSDQE
mouse	ILELNKRMSAVEHLLVHLENTVLPPSAQEPTVETHPSADTEETLRRRLEELTSNIGSS
dog	CLAG-----REPTADREEETLRRKLEELTCRVSDQD
mink (wt)	VRHL-----RNFPS
	. . * ***:***:***:***:***:***:***:***:***
	405
human	TSSEEEAKDEKAEPNRDKSVGPLPQADPEVGTAHQTNRQEKSPQDPGDPVQYNRTTDE
mouse	TSSEDET-----KPDGTFLLGGSPKVCTDTGHMETQERNPRSPGNPARPTKSTDE
dog	ASSEEA GSEEEGSDLARSPPSQDLPGAPEVCAAAGQTHGRDTPWGPQDLVQPGRTTDE
	:***: : * * : * : : : : : * * : : : :***
human	ELSELEDRVAVTASEVQQAEEVSDIESRIAALRAAGLTVKPSGKPRRKSNIPLFPRVA
mouse	ELSEMEDRVAMTASEVQQAEEISDIQSRIAALRAAGLTVKPSGKPRRKSIGIPLFPRVT
dog	ELLQLEDRVALTACEVQVVESEVSNISKIAALQAAGLTVKPSGKPRRKSNIPLFPRLA
	** : :***:***:***:***:***:***:***:***:***:***:***:***:***:***:***
human	GKLGKRPEDPNADPSSEAKA---MAVPYLLRRKFSNSLSKSGKDDDSF-DRKSVYRGSL
mouse	EKLDRIPKTPPADPDDQAKMPKATTAVPSLLRRKYSP--SSQGVDSGSF-DRKSVYRGSL
dog	GSYDQRFKDPNAEPSDE-----VTAPYLLRRKFSNSPKSQGRAADSARPSASAYRGSL
	. : * : * * : * : : * * : * : * * * * *
	590
human	TQRNPNAKGMASHTFAKPVVAHQ
mouse	TQRNPNGRRGTARHIFAKPVMAQQP
dog	TQRNPNSRKGVAASHFAKPVMTQQP
	*****:***:***:***:***:***:***:***:***:***

Fig. 2. MLPH protein alignment of human, mouse, dog and mink (wt). In light gray, the RAB27A binding domain (from residue 1 to 146); in dark gray the MYO5A Va binding domain (from residue 241 to 405), from the MYO5A Va domain to the end corresponds to the actin-binding domain (from residue 401 to 590). *, identical residues; ., residues with similar properties; -, deletion. In bold, italics and underlined the only residue that changes between wt and *Violet*. The residue number indicated in the beginning and at the end of each domain is based on the mouse sequence which is 590 amino acids long.

3.4. Analysis of the mRNA and protein sequences of the MLPH gene in mink

In order to investigate the effect of the SNP affecting the 5' splice site in intron 8, we decided to sequence the cDNA from wt (lung and kidney), *Violet* (only lung available) and *Silverblue* (lung and kidney)

animals. Different primer combinations were used in order to cover the region encompassed from the end of exon 7 to the beginning of exon 9 (see Table 2 for primer sequences).

The sequencing revealed that the MLPH transcripts from wt, *Violet* and *Silverblue* had retained part of intron 8 (see the light gray box in Fig. 4). In fact, the sequence from position 1 to position 2224 in intron

8 is spliced out in *wt* and *Violet* and the rest of the intron (581 bp) is retained in the cDNA. This retained sequence gives rise to a truncated protein in *wt* and *Violet* due to the introduction of a stop codon after position 177 of the intron, resulting in a MLPH protein 359 residues long (Fig. 2). In this retained intron we found 1 non-synonymous SNP between *wt* and *Violet* in position 19 of the intron 8 which results in a change from a Serine (S, neutral polar amino acid) in *wt* to an Asparagine (N, neutral non-polar amino acid) in *Violet*. In *Silverblue* the situation is even more complex as, surprisingly, the mRNA sequence lacks the entire exon 8, leading to a shift in the open reading frame (ORF). Therefore, the retained sequence of part of intron 8 introduces a stop codon at nt 147 resulting in a hypothetical protein consisting of 284 residues, where the last 49 residues are totally different from the *wt* and *Violet* phenotypes (Fig. 3).

When using the sequence of this partially retained intron to BLAST against the non-redundant database at National Center for Biotechnology Information (NCBI), we found homology to a MLPH transcript variant in ferret (*Mustela putorius furo*) and Giant panda (*Ailuropoda melanoleuca*).

When examining the different domains coded in the MLPH protein (Philipp et al., 2005), the truncated protein in *wt* and *Violet* mink results only in the lack of the last 28 residues of the MYO5A binding domain and the complete loss of the actin binding site. In *Silverblue*, the MLPH protein lacks the majority of the MYO5A Va binding domain and the complete actin binding domain. Additionally, the last 49 residues of the protein are very different between the *wt*/*Violet* mink (Fig. 3) and the other studied mammals.

3.5. Genetic test

The fixed polymorphisms between the *Violet* and *wt* found in the MLPH gene region (Fig. 4) can be readily used for genetic testing. For the SNPs, TaqMan assays (developed by Applied Biosystems) can be designed for the 5 bp insertion in *Violet* and the two deletions found in *Silverblue*, acrylamide and agarose electrophoresis systems can be applied respectively.

4. Discussion

In our previous study (Anistoroaei and Christensen, 2007) we mapped the MLPH gene, which is involved in the *Silverblue* phenotype in mink. The phenotype is similar to the dilution color in other species such as dog, cat, and chicken. As the resources available for the American mink (BAC library) allowed more investigations, we continued our study in order to clearly confirm and characterize the role of the MLPH gene in the expression of this phenotype. We started by re-genotyping the *Silverblue* family material from Anistoroaei and Christensen (2007) with newly developed microsatellite markers (RAN79, RAN80 and RAN81; (Table 1 and Supplementary Fig. 1)) and the results confirmed undoubtedly the previous association of the MLPH gene locus with this phenotype. We also genotyped a family

segregating for the *Violet* phenotype. This phenotype was chosen based on the fact that farming practices as well as literature indicate that *Violet* is a triple recessive phenotype clearly involving the *Silverblue* locus (MLPH, *pp*). The family was however non informative for the three markers.

Sequencing of (part of) the genomic region for the MLPH gene in the three coat color phenotypes (*wt*, *Violet* and *Silverblue*) revealed different SNPs, one small insertion between *wt* and *Violet* (CATTT in *wt* changes to GGGGTAGGAA in *Violet*) at position 5847 and a deletion of the whole intron 7 and part of intron 8 uniquely in *Silverblue*. Except for the two large deletions for the *Silverblue*, most of the SNPs and the 5 bp insertion have no functional consequences because they are located in untranslated regions or are synonymous. One not fixed non-synonymous SNP was found in exon 8. Furthermore, an interesting SNP was found at the splice donor site in exon 8/intron 8 of the *Violet* sequence. In order to investigate the phenomena further, we isolated RNA from *wt*, *Silverblue* and *Violet* animals and sequenced the MLPH transcript. Surprisingly, *Silverblue* lacks the entire exon 8 and has also retained part of the intron 8 in the coding region. This introduces a stop codon, leading to a truncated protein 284 residues long that is lacking almost all MYO5A Va and all actin-binding domains. In *wt* and *Violet* transcripts, the exon 8 is present but the part of intron 8 is also retained resulting in a protein 359 residues long (lacking the last 28 residues of the MYO5A Va and all actin-binding domains), in comparison to the 572 residues in humans, 590 in mouse and 581 in dog. The retention of part of intron 8 cannot be explained by the mutation in the acceptor splice site in intron 8 because the SNP it is only found in *Violet* animals.

When comparing the MYO5A Va domain region between the *wt* mink and the human–mouse–dog there is about 40% of similarity, in contrast with the RAB27A binding domain, where there is around 95% of similarity (Fig. 2). When comparing the actin-binding domain between the human, mouse and dog (mink lacks this domain) we can see 73.5% similarity. These results point towards the fact that the MYO5A Va domain is the lowest conserved domain of the 3 domains present in the MLPH. It is also in this domain that we find a deletion of the last 28 amino acids in mink. Moreover, a 28 amino acid insertion is found in mouse in this domain (Fig. 2). MLPH has been characterized as a linker protein in a tripartite protein complex formed by MYO5A Va and RAB27A. MLPH interacts with RAB27A via its terminal region (amino acids 1–146, light-gray region in Fig. 2) and with MYO5A Va via the central region (amino acids 241 to 405, dark-gray region in Fig. 2). RAB27A encodes a small Ras-like GTPase that seems to play a role in intracellular vesicle transport by controlling vesicle sorting, tethering, docking and fusion, while MYO5A is a protein that moves towards the end of actin filaments and functions in intracellular vesicle transport (i.e. melanosome transport) (Kuroda et al., 2003).

The complex RAB27A-MYO5A Va-MLPH is involved in the capture and short-range actin-based delivery of melanosomes to the melanocytes periphery. This transport is vital to subsequent delivery of the

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wt      ATDSLQVPCAQTFMDEPRAKDASPEAMVAPGCQPPPEEQMDSLSPVGQDTLTLCVPGES
Silverblue ATDSLQGG-----ASPGVSSSPPSIWPTWTP-----
*****                               .:* * . * . ::*

wt      RRTALGTAAGRSITGSEQLPQLADMDTSEDDGSPRAPHAAASHPCRGGGHTPSQSQ
Silverblue RRT-MA-----RAPGLPTLPPTLAE-----EEATPRPR-----
*** :. * ** * : **

wt      VRHLRNFPSC
Silverblue VR-----
**

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Fig. 3. Alignment of the 3' terminal part of the MLPH protein of *wt* and *Silverblue* mink. *, identical residues; :, residues with very similar properties, residues with similar properties; -, deletion.

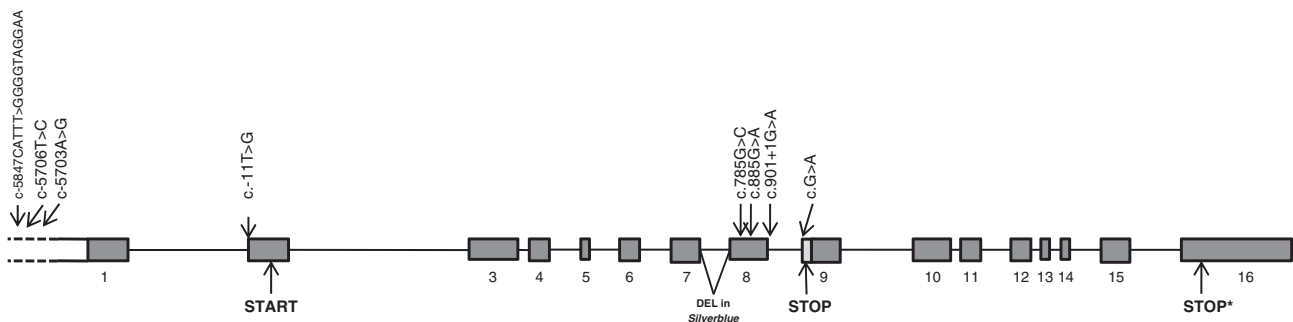


Fig. 4. *MLPH* genomic region. Polymorphisms between *wt* and *Violet*. Dark gray boxes are the in silico predicted exons. Dotted boxes indicate the UTRs. The STOP* has been in silico predicted. Results from our sequenced cDNA revealed a nonsyn SNP and a STOP codon within the retained part of intron 8 (light gray box). The figure also shows the deletion of intron 7 in the *Silverblue* mink.

melanosomes (containing the melanin pigment) into keratinocytes (Fukuda et al., 2002; Kuroda et al., 2003; Nagashima et al., 2002; Provance et al., 2002). A loss of any of the two domains (RAB27A or MYO5A Va), as is the case for the *Silverblue* phenotype, probably leads to a “recycling” of the melanosomes back to the melanocyte perinuclear area. Thus, the melanophilin with all its three domains is an essential element for proper melanosomes distribution in melanocytes.

Studies in mouse, using deletion mutant (DeltaAB) and point mutants (KA) which lack the actin-binding capacity, have shown that the actin-binding domain is also required for melanosome distribution in melanocytes, together with the RAB27A and MYO5A Va domains (Kuroda et al., 2003). In these studies, the authors conclude that the melanosome transport requires cooperation within the three domains. In contrast, a study of Li et al. (2005) showed that deletion of the actin-binding domain and Rab-binding domain of *MLPH* did not affect the activation of the ATPase, indicating that the MYO5A Va binding domain is sufficient for the activation of MYO5A Va. The results of our study in mink question the previous investigations by showing that the actin-binding domain seems not to be essential for the transport of melanosomes.

Additionally, transcriptome data derived from 4 different pooled tissues (liver, brain, skin and lung) of the American mink has recently been produced (Anistoroaei et al., 2012a). From this data we could identify both the full length transcript, which is transcribed down to exon 16 (like in other mammals) and a new short transcript (the only one identified in the present study) missing from exon 9 to exon 16, which confirms the findings of this study. Therefore, we cannot dismiss the possibility that the long variant including the actin-binding domain, which was identified in the transcriptome data (Anistoroaei et al., 2012a) still compensates for the actin binding function in other tissues than the ones investigated here (lung and kidney). I.e. the brain tissue included in the transcriptome project was not analyzed in the present study and with its known high gene expression activity it might contain the full length transcript.

In conclusion, *wt* and *Violet* are rather similar in the genomic and transcript sequences for the *MLPH* gene. Contrastingly, the *Silverblue* phenotype lacks intron 7 and part of intron 8 in the genomic sequence and the complete exon 8 at the cDNA level resulting in a very short and probably non-functional MYO5A Va binding domain. This deficiency would explain the defective distribution of melanosomes in melanocytes culminating in the dilute *Silverblue* coat color. Furthermore, our study confirms the previously generated information on mink genetics, indicating that more than one allele exists for the *Silver* locus (Clausen et al., 2012).

The two deletions in the *Silverblue MLPH* gene and the identified polymorphisms between *wt* and *Violet* provide the basis for designing genetic tests to select for *Silverblue* and *Violet* carriers respectively in American mink.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2013.05.047>.

Conflict of interest

The authors declare no conflicts of interest.

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References

- Anistoroaei, R., Christensen, K., 2007. Mapping of the silver gene in mink and its association with the dilution gene in dog. *Cytogenet. Genome Res.* 116, 316–318.
- Anistoroaei, R., ten Hallers, B., Nefedov, M., Christensen, K., de Jong, P., 2011. Construction of an American mink Bacterial Artificial Chromosome (BAC) library and sequencing candidate genes important for the fur industry. *BMC Genomics* 12, 354.
- Anistoroaei, R., Krogh, A.K., Christensen, K., 2013. A frameshift mutation in the *LYST* gene is responsible for the Aleutian colour and the associated Chédiak-Higashi syndrome in American mink. *Anim. Genet.* 44 (2), 178–183.
- Anistoroaei, R., Croft, L., Christensen, K., 2012a. An American mink transcriptome. *Proceedings of International Society of Animal Genetics*, July 15–20, Cairns, Australia, 144, p. P6000.
- Anistoroaei, R., Nielsen, V., Markakis, M.N., Karlsson-Mortensen, P., Jørgensen, C.B., Christensen, K., Fredholm, M., 2012b. A re-assigned American mink (*Neovison vison*) map optimal for genome-wide studies. *Gene* 511 (1), 66–72.
- Barral, D.C., Seabra, M.C., 2004. The melanosome as a model to study organelle motility in mammals. *Pigment Cell Res.* 17 (2), 111–118 (Apr).
- Bed'hom, B., et al., 2008. The lavender plumage colour phenotype in quail is caused by a large deletion in *MLPH* gene region. *Proceedings of the XXXI International Conference of the International Society for Animal Genetics*, July, Amsterdam, The Netherlands, pp. 15–20.
- Clausen, J., Hansen, B.K., Weiss, V., 2012. In: *Copenhagen Fur* (Ed.), Mink, bind2 (ISBN 879156672X, 9788791566721).
- Drögemüller, C., Philipp, U., Haase, B., Günzel-Apel, A.R., Leeb, T., 2007. A noncoding melanophilin gene (*MLPH*) SNP at the splice donor of exon 1 represents a candidate causal mutation for coat colour dilution in dogs. *J. Hered.* 98 (5), 468–473.
- Fukuda, M., Kuroda, T.S., Mikoshiba, K., 2002. Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport. *J. Biol. Chem.* 277, 12432–12436.
- Hume, A.N., Ushakov, D.S., Tarafder, A.K., Ferenczi, M.A., Seabra, M.C., 2007. Rab27a and MyoVa are the primary *Mlp* interactors regulating melanosome transport in melanocytes. *J. Cell Sci.* 120, 3111–3122.
- Ishida, Y., et al., 2006. A homozygous single-base deletion in *MLPH* causes the dilute coat colour phenotype in the domestic cat. *Genomics* 88, 698–705.
- Kuroda, T.S., Ariga, H., Fukuda, M., 2003. The actin-binding domain of Slac2-a/melanophilin is required for melanosome distribution in melanocytes. *Mol. Cell. Biol.* 23, 5245–5255.
- Li, X.D., Ikebe, R., Ikebe, M., 2005. Activation of myosin Va function by melanophilin, a specific docking partner of myosin Va. *J. Biol. Chem.* 280 (18), 17815–17822 (2005 May 6).
- Matesic, L.E., et al., 2001. Mutations in *Mlp*, encoding a member of the Rab effector family, cause the melanosome transport defects observed in leaden mice. *Proc. Natl. Acad. Sci. U. S. A.* 98, 10238–10243.
- Menasche, G., et al., 2003. Griscelli syndrome restricted to hypopigmentation results from a melanophilin defect (GS3) or a MYO5A F-exon deletion (GS1). *J. Clin. Invest.* 112 (3), 450–456.
- Mercer, J.A., Seperack, P.K., Strobel, M.C., Copeland, N.G., Jenkins, N.A., 1991. Novel myosin heavy chain encoded by murine dilute coat colour locus. *Nature* 349, 709–713.

- Nagashima, K., et al., 2002. Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions. *FEBS Lett.* 517, 233–238.
- Nascimento, A.A., Roland, J.T., Gelfand, V.I., 2003. Pigment cells: a model for the study of organelle transport. *Annu. Rev. Cell Dev. Biol.* 19, 469–491.
- Passeron, T., Mantoux, F., Ortonne, J.P., 2005. Genetic disorders of pigmentation. *Clin. Dermatol.* 23 (1), 56–67.
- Philipp, U., Hamann, H., Mecklenburg, L., Nishino, S., Mignot, E., Günzel-Apel, A.R., Schmutz, S.M., Leeb, T., 2005. Polymorphisms within the canine MLPH gene are associated with dilute coat colour in dogs. *BMC Genet.* 6, 34 (Jun 16).
- Provance Jr., D.W., James, T.L., Mercer, J.L., 2002. Melanophilin, the product of the leaden locus, is required for targeting of myosin-Va to melanosomes. *Traffic* 3, 124–132.
- Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132, 365–386.
- Vaez, M., Follett, S.A., Bed'hom, B., Gourichon, D., Tixier-Boichard, M., Burke, T., 2008. A single point-mutation within the melanophilin gene causes the lavender plumage colour dilution phenotype in the chicken. *BMC Genet.* 9, 7 (15).
- Wilson, S.M., et al., 2000. A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proc. Natl. Acad. Sci.* 97, 7933–7938.